Selective Cholinesterase Inhibitory Activities of a New Monoterpene Diglycoside and Other Constituents from *Nelumbo nucifera* Stamens

Hyun Ah JUNG,^a Yu Jung JUNG,^a Sook Kyung HYUN,^a Byung-Sun MIN,^b Dong-Wook KIM,^c Jee H. JUNG,^d and Jae Sue CHOI^{*a,e}

^a Division of Food Science and Biotechnology, Pukyong National University; Busan 608–737, Republic of Korea; ^b College of Pharmacy, Catholic University of Daegu; Gyeongbuk 712–702, Republic of Korea; ^c Division of Applied Bioscience, Mokpo National University; 61 Chonggye-myeon, Mu-an-gun, Jeonnam 534–792, Republic of Korea; ^d College of Pharmacy, Pusan National University; Busan 609–735, Republic of Korea; and ^e Blue-Bio Industry RIC; 995 Eomgwangno Busanjin-gu, Busan 614–714, Republic of Korea. Received July 17, 2009; accepted October 22, 2009

A new β -cyclogeraniol diglycoside (**5**), along with four known components, cycloartenol (**1**), *p*-hydroxybenzoic acid (**2**), vanilloloside (**3**), and 5'-*O*-methyladenosine (**4**), were first isolated from the *n*-BuOH fraction of *Nelumbo nucifera* stamens. The chemical structure of **5** was elucidated as 1-hydroxymethyl-2,6,6-trimethyl-1-cyclohexene 9-*O*- β -D-glucopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranoside (nuciferoside) on the basis of chemical and spectroscopic evidence, including 1D, 2D NMR, and MS. The anti-Alzheimer effects of **1**–**5** were evaluated *via* the acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and β -site amyloid precursor protein (APP) cleaving enzyme 1 (BACE1) inhibition assays. Compounds **1**–**3** and **5** showed good and noncompetitive inhibition against AChE with IC₅₀ values of 11.89, 20.07, 4.55, and 3.20 μ M and K_i values of 15.71, 25.44, 7.76, and 5.76 μ M, respectively. Compounds **1**, **2**, and **5** also possessed BChE inhibitory activities with IC₅₀ values of 13.93, 62.29, 205.78, and 83.06 μ M, respectively. The selectivity index (SI) values of **1**, **2**, **3**, and **5**, calculated from IC₅₀ values of BChE and AChE, were 1.2, 3.1, 45.7, and 26.0. However, all isolated compounds lacked BACE1 inhibition up to 100 μ M. Therefore, *N. nucifera* stamens-derived compounds could potentially exert their primary anti-Alzheimer effects as AChE inhibitors rather than BACE1 inhibitors.

Key words *Nelumbo nucifera*; β -cyclogeraniol diglycoside; nuciferoside; cholinesterase inhibition; kinetic parameter

Nelumbo nucifera GAERTN., the family of Nelumbonaceae, is a perennial, rhizomatous, aquatic plant distributed throughout Asia, including India, China, and Egypt. The plant is comprised of: membranous, peltate, orbicular, and concave leaves; nut-contained and ovoid fruits; black, hard, and ovoid seeds; nodal roots. In Korea, China and India, various parts of *N. nucifera* have been used in foodstuffs and traditional medicine for the treatment of diarrhea, gastritis, insomnia, nervous prostration and as a haemostatic.^{1–3} In particular, numerous biological activities, including antioxidant,^{4,5} anti-human immunodeficiency virus (HIV),⁶ antihyperlipidemic,¹ hepatoprotective, and antiobesity effects^{7–9} have been reported from the leaves of *N. nucifera*. In addition, Mukherjee *et al.* reported that *N. nucifera* rhizomes possessed diverse bioactivities, including antibacterial,¹⁰ antidiarrhoeal,¹¹ diuretic,¹² antifungal,¹³ antipyretic,¹⁴ anti-inflammatory,¹⁵ and antidiabetic.^{16,17} Additionally, the rhizomes have been reported to exhibit antihypertensive¹⁸ and memory enhancing activities.¹⁹ Regarding the seeds, several reports exist on their hepatoprotective,²⁰ antioxidant,²¹ antifertility,²² and antipyretic activities,²³ as well as their inhibitory effects on pulmonary fibrosis.²⁴ Recently, *N. nucifera* semen extract was reported to improve memory in rats with scopolamine-induced dementia through the induction of choline acetyltransferase expression and inhibition of acetylcholinesterase (AChE).²⁵ Even though individual parts of this plant have been the target of distinct biological and pharmacological research, there are limited studies of the stamens, with the exception of research focusing upon the antioxidant and rat lens aldose reductase (RLAR) inhibition^{26–28} and anti-allergic activities²⁹ attributed to the flavonoids.

Alzheimer's disease (AD) is a neurodegenerative disease and the most frequent and predominant cause of dementia in

the elderly, provoking progressive cognitive decline, psycho-behavior disturbances, memory loss, the presence of senile plaques, neurofibrillary tangles, and a decrease in cholinergic transmission.^{30,31} Although the pathogenesis of AD is complicated and involved in numerous pathways, two major hypotheses are currently under consideration regarding the molecular mechanism, the cholinergic hypothesis and the amyloid cascade hypothesis. Thus, the focus herein is upon inhibitors of select cholinesterases (ChEs) to alleviate cholinergic deficits and improve neurotransmission and β -site amyloid precursor protein (APP) cleaving enzyme 1 (BACE1; aspartyl protease, β -secretase, and memapsin2) inhibitors to preclude formation and accumulation of amyloid β peptide (A β). Pursuant to this, both could then be established as viable therapeutic targets for AD.^{31–34}

Given that numerous promising pharmaceuticals and nutraceuticals are constantly isolated from effective natural products, and that a previous study from this lab indicated potent antioxidant and RLAR inhibitory activities of *N. nucifera* stamens, this plant was chosen for ongoing investigations into anti-AD activities. The present work deals with the isolation and characterization of a new β -cyclogeraniol diglycoside (**5**), and four known compounds: cycloartenol (**1**); *p*-hydroxybenzoic acid (**2**); vanilloloside (**3**); 5'-*O*-methyladenosine (**4**). Furthermore, the inhibitory activities of **1**–**5** toward ChEs, including AChE and butyrylcholinesterase (BChE), and BACE1 were evaluated.

MATERIALS AND METHODS

General Experimental Procedures Optical rotation was determined on a Perkin-Elmer 341 MC polarimeter (Norwalk, CT, U.S.A.). The ¹H- and ¹³C-NMR, and distor-

* To whom correspondence should be addressed. e-mail: choijs@pknu.ac.kr.

tionless enhancement by polarization transfer (DEPT) spectra were determined using a JEOL JNM ECP-400 spectrometer (Tokyo, Japan) at 400 MHz for ^1H and 100 MHz for ^{13}C . The chemical shifts were referenced to residual solvent peaks (CDCl_3 , $\text{DMSO}-d_6$, and pyridine- d_5). The EI-MS data were collected on a GC-MS QP-5050A spectrometer (Shimadzu, Kyoto, Japan). The LR and HR FAB-MS data were assessed using a JEOL JMS-HX110/110A spectrometer (Tokyo, Japan). Column chromatography was conducted using silica (Si) gel 60 (70–230 mesh, Merck, Darmstadt, Germany), RP-18 Lichroprep (40–63 μm , Merck, Germany), and Amberlite MB-3 (Sigma Co., St. Louis, MO, U.S.A.). TLC (thin layer chromatography) was conducted on precoated Merck Kieselgel 60 F_{254} plates (20 $\times$ 20 cm, 0.25 mm) and a RP-18 F_{254s} plates (5 $\times$ 10 cm, Merck, Darmstadt, Germany), using 50% H_2SO_4 as a spray reagent.

Chemicals and Reagents Electric-eel AChE (EC 3.1.1.7), horse-serum BChE (EC 3.1.1.8), acetylthiocholine iodide (ACh), butyrylthiocholine chloride (BCh), 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB), quercetin, and galanthamine were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). BACE1 fluorescence resonance energy transfer (FRET) assay kit (β -Secretase) was purchased from the Pan-Vera Co. (Madison, WI, U.S.A.). All chemicals and solvents used were purchased from E. Merck, Fluka, and Sigma & Aldrich Co., unless stated otherwise.

Plant Materials The stamens of *N. nucifera* were purchased from a local retailer and authenticated by Prof. D. W. Kim at Mokpo National University (Jeonnam Province, Republic of Korea). A voucher specimen (No. 20040420) was deposited in the laboratory of Prof. J. S. Choi.

Extraction, Fractionation, and Isolation The stamens (3.4 kg) of *N. nucifera* were refluxed in methanol (MeOH) for 3 h (91 $\times$ 3). The total filtrate was then concentrated to dryness *in vacuo* at 40 $^\circ\text{C}$ to render the MeOH extract (560 g). This extract was suspended in distilled water (H_2O) and then successively partitioned between methylene chloride (CH_2Cl_2), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH), to yield CH_2Cl_2 (330 g), EtOAc (20 g), *n*-BuOH (66 g) fractions, respectively, as well as an H_2O residue (140 g). A portion of the *n*-BuOH fraction (26.6 g) was first chromatographed over a Si gel column (100 mm i.d. $\times$ 80 cm), using a mixed solvent system of EtOAc–MeOH (20:1 $\rightarrow$ 10:1 $\rightarrow$ 5:1 $\rightarrow$ 0:1, gradient) to afford thirteen fractions (BF01–BF13). Repeated column chromatography of fraction 1 (BF01, 1.5 g) was performed over a Si gel column (20 mm i.d. $\times$ 20 cm) with *n*-hexane–EtOAc (15:1 $\rightarrow$ 0:1, gradient) to obtain cycloartenol (**1**, 25 mg) and *p*-hydroxybenzoic acid (**2**, 13 mg). Repeated column chromatography of combined fractions 2 and 3 (BF02/03, 9 g) was performed over a Si gel column (60 mm i.d. $\times$ 60 cm) with EtOAc–MeOH– H_2O (40:1:1) yielding twelve fractions (BF02/03-01–BF02/03-12). Fraction 12 (BF02/03-12, 1.5 g) was chromatographed over an RP-18 gel column (20 mm i.d. $\times$ 20 cm) with 50% MeOH, yielding eight fractions (BF02/03-12-1–BF02/03-12-8). Fraction 4 (BF02/03-12-4, 300 mg) was chromatographed over a Si gel column (20 mm i.d. $\times$ 20 cm) with CH_2Cl_2 –MeOH– H_2O (65:35:10) to obtain vanilloloside (**3**, 12 mg) and 5'-*O*-methyladenosine (**4**, 15 mg). A precipitate of fraction 5 (BF02/03-12-5, 250 mg) was purified using an HPLC system consisting of a 307 pump (Gilson, F-95400 Villiers Le Bel, France), RI detector

Fig. 1. Structure of Compounds **1**–**5** from *N. nucifera* Stamens

Table 1. NMR Spectral Data of **5** Isolated from *N. nucifera* Stamens

No.	^1H	^{13}C
1		134.5
2		134.3
3	1.81 (2H, m)	32.9
4	1.47 (2H, m)	19.6
5	1.34 (2H, m)	39.6
6		34.2
7	1.12 (3H, s)	28.7
8	1.11 (3H, s)	28.6
9	4.85 (1H, d, $J=12.5$ Hz) 4.37 (1H, dd, $J=12.5, 5.2$ Hz)	70.2
10	1.77 (3H, s)	20.2
1'	4.75 (1H, d, $J=7.8$ Hz)	104.4
2'	3.92 (1H, m)	74.9
3'	4.24 (1H, m)	78.4
4'	4.24 (1H, m)	71.6
5'	4.18 (1H, m)	77.4
6'	4.85 (1H, m) 4.11 (1H, m)	66.0
1''	5.17 (1H, d, $J=7.8$ Hz)	105.5
2''	4.05 (1H, m)	75.2
3''	4.24 (1H, m)	78.4
4''	4.05 (1H, m)	71.9
5''	3.92 (1H, m)	78.4
6''	4.52 (1H, dd, $J=12.3, 2.2$ Hz) 4.34 (1H, dd, $J=12.3, 4.9$ Hz)	62.7

(Shodex RI-101, Showa Denko, Kanagawa, Japan), and a Waters Spherisorb column (Semi-Prep column, 10 $\times$ 250 mm, ODS2) in 70% MeOH to obtain the cyclocitral-type monoterpen diglycoside, nuciferoside (**5**, 90 mg). The isolated compound (**5**) was identified by chemical and spectroscopic analysis, including 1D, 2D NMR, and MS; compounds **1**,^{35,36} **2**,³⁷ **3**,³⁸ and **4**³⁹ were characterized by comparison with published data (Fig. 1).

Nuciferoside (5) White amorphous powder; $[\alpha]_D^{20}$ -40° ($c=0.4$, MeOH); EI-MS m/z (%), relative intensity): 478 $[\text{C}_{22}\text{H}_{38}\text{O}_{11}]^+$ (0.16), 316 $[\text{M}-\text{Glc}]^+$ (0.16), 154 $[\text{M}-\text{Glc}-\text{Glc}]^+$ (32.6), 136 $[\text{M}-\text{Glc}-\text{Glc}-\text{H}_2\text{O}]^+$ (36.7); positive LR FAB-MS m/z : 501.2 $[\text{M}+\text{Na}]^+$; negative LR FAB-MS m/z : 477.25 $[\text{M}-\text{H}]^-$; HR FAB-MS m/z : 501.2308 (Calcd for $\text{C}_{22}\text{H}_{38}\text{O}_{11}\text{Na}$: 501.2336); ^1H -NMR (400 MHz, pyridine- d_5) δ : given in Table 1; ^{13}C -NMR (100 MHz, pyridine- d_5) δ : given in Table 1.

Determination of Sugar in 5 Compound **5** was refluxed with 2N HCl/dioxane (1:1, 2 ml) for 2 h. The mixture was

extracted with EtOAc (5 ml×3 times). The residual water layer was neutralized with Amberlite MB-3 and dried to obtain a residue. The residue was dissolved in pyridine (1 ml), and 0.1 M L-cysteine methyl ester hydrochloride in pyridine (2 ml) was added. The mixture was maintained at 60 °C for 1.5 h. After drying the reaction mixture *in vacuo*, the residue was trimethylsilylated with hexamethyldisilazane-trimethylchlorosilane (0.1 ml) at 60 °C for 1 h. The mixture was partitioned between hexane and H₂O (0.3 ml each), and the hexane extract was analyzed by GC (column, DB-1, 0.25 mm i.d.×30 m; column temperature, 50—230 °C, 15 °C/min, 230 °C for 18 min; carrier gas, He). The sugar derivatives obtained exhibited a retention time of 21.30 min, identified with that of authentic D-glucose. Under these conditions, a derivative of L-glucose displayed a retention time of 22.00 min.⁴⁰⁾

In Vitro ChEs Enzyme Assay The inhibitory activities of the ChEs were measured using the spectrophotometric method developed by Ellman *et al.*⁴¹⁾ ACh and BCh were used as the substrates to assay the inhibitions of AChE and BChE, respectively. The reaction mixture contained: 140 μ l of sodium phosphate buffer (pH 8.0); 20 μ l of tested sample solution (final concentration (f.c.) 100 μ M for the compounds); and 20 μ l of either AChE or BChE solution, which were mixed and incubated for 15 min at room temperature. All tested samples and the positive control (galanthamine) were dissolved in 10% analytical grade dimethyl sulfoxide (DMSO). The reactions were started with the addition of 10 μ l of DTNB and 10 μ l of either ACh or BCh, respectively. The hydrolysis of ACh or BCh was monitored by following the formation of the yellow 5-thio-2-nitrobenzoate anion at 412 nm for 15 min, which resulted from the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of either ACh or BCh, respectively. All reactions were performed in triplicate and recorded in 96-well microplates, using VERSA max (Molecular Devices, Sunnyvale, CA, U.S.A.). Percent inhibition was calculated from $(1 - S/E) \times 100$, where E and S were the respective enzyme activities without and with the tested sample, respectively. The ChEs inhibitory activity of each sample was expressed in terms of the IC₅₀ value (μ M required to inhibit the hydrolysis of the substrate, ACh or BCh, by 50%), as calculated from the log-dose inhibition curve.

Kinetic Parameters of 1—3 and 5 in AChE Inhibition

In order to determine the inhibition mechanism, AChE inhibition was evaluated by monitoring the effects of different concentrations of substrates (0.6, 0.3, and 0.1 mM for **1**, **3**, and **5**; 0.6, 0.4, and 0.2 mM for **2**), respectively. The reaction mixture consisted of the aforementioned ChEs assay method: 140 μ l of sodium phosphate buffer (pH 8.0); 20 μ l of tested sample solution (f.c. 100 μ M for the compounds); 20 μ l of either AChE solution, mixed and incubated for 15 min at room temperature. The reactions were initiated upon addition of 10 μ l of DTNB and 10 μ l of either ACh. All tested samples and the positive control (galanthamine) were dissolved in 10% analytical grade DMSO. Inhibition constants (K_i) were determined by interpretation of the Dixon plot, where the value of the x-axis implies $-K_i$.⁴²⁾

In Vitro BACE1 Enzyme Assay The assay was carried out according to the supplied manual with select modifications. Briefly, a reaction solution which consists of 10 μ l of

assay buffer (50 mM sodium acetate, pH 4.5), 10 μ l of BACE1 (1.0 U/ml), 10 μ l of the substrate (750 nM Rh-EVNL-DAEFK-Quencher in 50 mM ammonium bicarbonate), and 10 μ l of the tested samples (f.c. 100 μ M for compounds) dissolved in 10% DMSO was incubated for 60 min at 25 °C in the dark. The proteolysis of two fluorophores (Rh-EVNL-DAEFK-Quencher) by BACE1 was monitored by the formation of the fluorescent donor (Rh-EVNL), which increases in fluorescence wavelengths at 530—545 nm (excitation) and 570—590 nm (emission), respectively. Fluorescence was measured with a microplate spectrofluorometer (Spectralmax Gemini XPS, Molecular devices, Sunnyvale, CA, U.S.A.). The mixture was irradiated at 545 nm and the emission intensity was recorded at 585 nm. Percent inhibition (%) was obtained by the following equation: % inhibition = $[1 - (S_{60} - S_0)/(C_{60} - C_0)] \times 100$, where C_{60} was the fluorescence of the control (enzyme, buffer, substrate) after 60 min of incubation, C_0 was the initial fluorescence of the control, S_{60} was the fluorescence of the tested samples (enzyme, sample solution, substrate) after 60 min of incubation, and S_0 was the initial fluorescence of the tested samples. To allow for the quenching effect of the samples, the sample solution was added to reaction mixture C, and any reduction in fluorescence by the sample was then investigated. The BACE1 inhibitory activity of each sample was expressed in terms of the IC₅₀ value (μ M required to inhibit the proteolysis of the substrate; BACE1, by 50%), as calculated from the log-dose inhibition curve. Quercetin was used as a positive control.

Statistics The Kruskal–Wallis test and the Mann–Whitney *U* test were used to determine the statistical significance of differences between values for various experimental and control groups. Data were expressed as the mean $\pm$ S.E.M. in triplicate.

RESULTS AND DISCUSSION

Recently, the rhizome and semen of *N. nucifera* have been reported to possess enhancing effects on memory and learning¹⁹⁾ and anti-amnesic effects in rats with scopolamine-induced dementia, respectively.²⁵⁾ To the best of the author's knowledge, *N. nucifera* stamens have yet to be studied in the context of anti-AD activity. In addition, phytochemical researches on polar fractions such as *n*-BuOH are limited. Therefore, focus was upon characterization of other intriguing components from the *N. nucifera* stamens, with anti-Alzheimer effects evaluated *via* AChE, BChE, and BACE1 inhibitory assays.

Likewise, a previous study revealed that the rhizome and semens of *N. nucifera* showed AChE inhibition.^{19,25,43)} In this lab's preliminary study, the MeOH extract of *N. nucifera* stamens exerted good ChEs inhibition: IC₅₀ values of 40.81 μ g/ml for AChE; 61.61 μ g/ml for BChE. Thus, bioactive-guided fractionation of the extract was performed to obtain the CH₂Cl₂, EtOAc, *n*-BuOH, and H₂O fractions. Although all bioactive data were not shown, the EtOAc and *n*-BuOH fractions exhibited slightly higher AChE inhibitory activities over other fractions, with respective IC₅₀ values of 33.58 and 24.73 μ g/ml. Since the EtOAc fraction was reported to contain numerous flavonoids,^{26,27)} and it is well known that these flavonoids might attribute to their ChEs inhibition,^{44,45)} detailed phytochemical investigations and

anti-AD studies on the *n*-BuOH fraction were carried out; the *n*-BuOH fraction also harbors AChE activities and lacks substantial research.

Repeated column chromatography of the *n*-BuOH fraction was accomplished to yield a new β -cyclogeraniol diglycoside (**5**), along with four known components **1**–**4** (Fig. 1). Through comparison of published NMR and EI-MS data, the structures of the known compounds were identified as cycloartenol (**1**),^{35,36} *p*-hydroxybenzoic acid (**2**),³⁷ vanilloside (**3**),³⁸ and 5'-*O*-methyladenosine (**4**).³⁹ This is the first report of isolation of these four, known compounds **1**–**4** from this plant.

Compound **5** was obtained as a white amorphous powder. The positive HR FAB-MS of **5** afforded a quasi-molecular ion peak at m/z 501.2308 $[M+Na]^+$, with a molecular formula of $C_{22}H_{38}O_{11}$. The 1H -NMR spectrum (Table 1) showed signals for: three methylenes at δ 1.34, 1.47, and 1.81 (each 2H, m); a hydroxymethyl at δ 4.85 (d, $J=12.5$ Hz) and 4.37

(dd, $J=12.5, 5.2$ Hz); three tertiary methyls at δ 1.11, 1.12, and 1.77 (each 3H, s), assigned to a β -cyclogeraniol.⁴⁶ This observation was further confirmed by ^{13}C -NMR spectrometric assignments (Table 1), coupled with the DEPT and heteronuclear multiple quantum correlation (HMQC) spectral data: two olefinic carbons at δ 134.3 and 134.5; one hydroxymethyl carbon at δ 70.2; three methyl carbons at δ 28.6, 28.7, and 20.2; three hydrocarbons at δ 19.6, 32.9, and 39.6; one quaternary carbon at δ 34.2. The respective heteronuclear multiple bond connectivity (HMBC) correlations of δ_H 1.81 (H-3) and δ_H 1.12 (H-7) to δ_C 134.3 (C-2)/134.5 (C-1) were observed. The hydroxymethyl group could be located at C-1 due to correlation between the signals of δ_H 4.85/4.37 (H-9) and δ_C 134.3 (C-2)/134.5 (C-1)/34.2 (C-6) in the HMBC spectrum. In addition, the 1H - 1H correlation spectroscopy (COSY) spectrum of **5** showed that the H-3 signal (δ 1.81) was coupled with the H-4 (δ 1.47), and in turn the H-4 signal was coupled with the H-5 (δ 1.34), indicating a three-methylene spin system in close proximity. However, the 1H -NMR contained signals for two anomeric protons at δ 5.17 and 4.75, and twelve oxygenated protons due to two hexose units, in combination with the ^{13}C -NMR spectrum. Upon acid hydrolysis, **5** yielded monosaccharide units identified by co-TLC (EtOAc–MeOH–H₂O–AcOH, 65:20:15:15) with an authentic sample (glucose). The absolute configuration was determined as D-glucose by GC analysis.⁴⁷ The configurations of the glycosidic linkages for the glucopyranoside units were determined as the β form on the basis of the $J_{1,2}$ values of the anomeric protons of 7.8 Hz (δ 5.17) and 7.8 Hz (δ 4.75). Sugar connectivity was determined on the basis of correlations between the δ_H 4.75 (H-1') and δ_C 70.2 (C-9), and δ_H 5.17 (H-1'') and δ_C 66.0 (C-6'), supporting the

Table 2. Anti-Alzheimer Activities of **1**–**5** from *N. nucifera* Stamens

Compounds	AChE ^{a)}	BChE ^{b)}	SI ^{c)}	BACE1 ^{d)}
1	11.89 $\pm$ 1.91	13.93 $\pm$ 1.65	1.2	>100
2	20.07 $\pm$ 0.07	62.29 $\pm$ 1.18	3.1	>100
3	4.55 $\pm$ 0.72	205.78 $\pm$ 0.79	45.7	>100
4	>100	>100	—	>100
5	3.20 $\pm$ 0.22	83.06 $\pm$ 0.80	26.0	>100
Galanthamine ^{e)}	0.02 $\pm$ 0.00	0.87 $\pm$ 0.07		
Quercetin ^{f)}				7.15 $\pm$ 0.45

a), b), d) Final concentrations of test samples were 100 μ M for test compounds, dissolved in 10% DMSO; 50% inhibition concentrations (IC_{50} , μ M) are expressed as the mean $\pm$ S.E.M. of triple experiments. c) SI: selectivity index (BChE/AChE). e) Galanthamine and f) quercetin were reference compounds in the ChEs and BACE1 assays.

Fig. 2. Dixon Plots for Inhibition of Compounds **1**–**3**, and **5** towards AChE in the Presence of Different Concentrations of Substrate

The reaction was performed in the presence of various concentrations of compounds. Results are expressed as means $\pm$ S.E.M. in triplicate. (A) Cycloartenol (**1**) [0.6 (●), 0.3 (○), 0.1 (▼) mM of ACh], $K_i=15.71$ μ M. (B) *p*-Hydroxybenzoic acid (**2**) [0.6 (●), 0.4 (○), 0.3 (▼) mM of ACh], $K_i=25.44$ μ M. (C) Vanilloside (**3**) [0.6 (●), 0.3 (○), 0.1 (▼) mM of ACh], $K_i=7.76$ μ M. (D) Nuciferoside (**5**) [0.6 (●), 0.3 (○), 0.1 (▼) mM of ACh], $K_i=5.76$ μ M.

presence of a gentiobiose. The structure of **5** was thus determined to be a new β -cyclogeraniol diglycoside [1-hydroxymethyl-2,6,6-trimethyl-1-cyclohexene 9-*O*- β -D-glucopyranosyl-(1 $\rightarrow$ 6)- β -D-glucopyranoside], and named nuciferoside.

The anti-Alzheimer effects of **1**–**5** from *N. nucifera* stamens were evaluated *via* the AChE, BChE, and BACE1 inhibition assays. As shown in Table 2, **1**–**3** and **5** exhibited good AChE inhibitory activities with IC₅₀ values of 11.89, 20.07, 4.55, and 3.20 μ M, respectively, as compared with a positive control, galanthamine (IC₅₀=0.02 μ M). In the BChE inhibitory assay, **1**, **2**, and **5** exhibited inhibitory activities with IC₅₀ values of 13.93, 62.29, and 83.06 μ M, as compared with galanthamine (IC₅₀=0.87 μ M). The selectivity index (SI) values of **1**, **2**, **3**, and **5**, calculated from IC₅₀ values of BChE and AChE, were 1.2, 3.1, 45.7 and 26.0 (Table 2). Although some research has been performed on ChEs inhibitory activities of monoterpenes^{48–50} and tetracyclic steroidal alkaloids,^{51,52} this is the first reports on the ChEs inhibitory activities of **1**–**3** and **5**.

In order to determine the type of AChE inhibition, kinetic analysis of **1**–**3** and **5** was carried out by monitoring the effects of three different concentrations of ACh as a substrate (0.6, 0.3, and 0.1 mM for **1**, **3**, and **5**; 0.6, 0.4 and 0.2 mM for **2**) by Dixon plots. As demonstrated in Figs. 2A–2D, the manner of inhibition of **1**–**3** and **5** was noncompetitive with *K_i* values of 15.71, 25.44, 7.76, and 5.76 μ M, respectively, as compared with a competitive inhibitor, galanthamine.⁵³ However, **1**–**5** showed no BACE1 inhibition up to 100 μ M.

In conclusion, a new β -cyclogeraniol diglycoside, nuciferoside (**5**), along with four known compounds, cycloartenol (**1**), *p*-hydroxybenzoic acid (**2**), vanilloloside (**3**), and 5'-*O*-methyladenosine (**4**), were isolated from the *n*-BuOH fraction of *N. nucifera* stamens for the first time. To further delineate their potential regarding prevention and treatment of AD, AChE, BChE, and BACE1 inhibition assays were carried out. As a result, **1**–**3** and **5** showed good and noncompetitive AChE inhibitory activities while **1**, **2**, and **5** exhibited BChE inhibitory activities. According to the ChEs assay results, the authors contend that **1**–**3** and **5** are possible ChEs inhibitors with potential for treatment of AD. Therefore, *N. nucifera* stamens-derived compounds could potentially exert their anti-Alzheimer effects primarily as AChE inhibitors rather than BACE1 inhibitors.

Acknowledgements This study was supported by the Technology Development Program for Agriculture and Forestry (TDPAF), Ministry of Agriculture and Forestry, Republic of Korea; Pukyong National University under the 2009 Post-Doc. Program; and Blue-Bio Industry RIC at Dongeui University as a RIC program under Ministry of Knowledge Economy and Busan city.

REFERENCES

- La Cour B., Mflgaard P., Yi Z., *J. Ethnopharmacol.*, **46**, 125–129 (1995).
- Sridhar K. R., Bhat R., *J. Agric. Technol.*, **3**, 143–155 (2007).
- Kim J. G., "Illustrated Natural Drugs Encyclopedia," Vol. II, Namsandang, Korea, 1997, p. 95.
- Cho E. J., Yokozawa T., Rhyu D. Y., Kim S. C., Shibahara N., Park J. C., *Phytochemistry*, **10**, 544–551 (2003).
- Wu M. J., Wang L., Weng C. Y., Yen J. H., *Am. J. Chin. Med.*, **31**, 687–982 (2003).
- Kashiwada Y., Aoshima A., Ikeshiro Y., Chen Y. P., Furukawa H., Itoigawa M., Fujioka T., Misashi K., Cosentino M. L., Morris-Natschke S. L., Lee K. H., *Bioorg. Med. Chem.*, **13**, 443–448 (2005).
- Ono Y., Hattori E., Fukaya Y., Imai S., Ohizumi Y., *J. Ethnopharmacol.*, **106**, 238–244 (2006).
- Ohkoshi E., Miyazaki H., Shindo K., Watanabe H., Yoshida A., Yajima H., *Planta Med.*, **73**, 1255–1259 (2007).
- Lin M. C., Kao S. H., Chung P. J., Chan K. C., Yang M. Y., Wang C. J., *J. Agric. Food Chem.*, **57**, 5925–5932 (2009).
- Mukherjee P. K., Balasubramanian R., Saha K., Pal M., Saha B. P., *Indian Drugs*, **32**, 274–276 (1995).
- Mukherjee P. K., Das J., Balasubramanian R., Saha K., Pal M., Saha B. P., *Indian J. Pharm.*, **27**, 262–364 (1995).
- Mukherjee P. K., Das J., Saha K., Pal M., Saha B. P., *Phytother. Res.*, **10**, 424–425 (1996).
- Mukherjee P. K., Giri S. N., Saha K., Pal M., Saha B. P., *Indian J. Microbiol.*, **35**, 327–330 (1995).
- Mukherjee P. K., Das J., Saha K., Giri S. N., Pal M., Saha B. P., *Indian J. Exp. Biol.*, **34**, 275–276 (1996).
- Mukherjee P. K., Saha K., Das J., Pal M., Saha B. P., *Planta Med.*, **63**, 367–369 (1997).
- Mukherjee P. K., Pal S. R., Saha K., Saha B. P., *Phytother. Res.*, **9**, 522–524 (1995).
- Mukherjee P. K., Saha K., Pal M., Saha B. P., *J. Ethnopharmacol.*, **58**, 207–213 (1997).
- Shoji N., Umeyama H., Saito N., Iuchi A., Takemoto T., Kajiura A., Ohizumi Y., *J. Nat. Prod.*, **50**, 773–774 (1987).
- Yang W. M., Shim K. J., Choi M. J., Park S. Y., Choi B. J., Chang M. S., Park S. K., *Neurosci. Lett.*, **443**, 104–107 (2008).
- Sohn D. H., Kim Y. C., Oh S. H., Park E. J., Li X., Lee B. H., *Phytomedicine*, **10**, 165–169 (2003).
- Rai S., Wahile A., Mukherjee K., Saha B. P., Mukherjee P. K., *J. Ethnopharmacol.*, **104**, 322–337 (2006).
- Mazumder U. K., Gupta M., Pramanik G., Mukhopadhyay R. K., Sarkar S., *Indian J. Exp. Biol.*, **30**, 533–534 (1992).
- Xin L. Z., "The Pharmacology of Chinese Herbs," ed. by Huang K. C., CRC Press Inc., U.S.A., 1999, p. 192.
- Xiao J. H., Zhang J. H., Chen H. L., Feng X. L., Wang J. L., *Planta Med.*, **71**, 225–305 (2005).
- Oh J. H., Choi B. J., Chang M. S., Park S. K., *Neurosci. Lett.*, **461**, 41–44 (2009).
- Jung H. A., Kim J. E., Chung H. Y., Choi J. S., *Arch. Pharm. Res.*, **26**, 279–285 (2003).
- Hyun S. K., Jung Y. J., Chung H. Y., Jung H. A., Choi J. S., *Arch. Pharm. Res.*, **29**, 287–292 (2006).
- Lim S. S., Jung Y. J., Hyun S. K., Lee Y. S., Choi J. S., *Phytother. Res.*, **20**, 825–830 (2006).
- Shim S. Y., Choi J. S., Byun D. S., *J. Microbiol. Biotechnol.*, **19**, 155–602 (2009).
- Scarpini E., Scheltens P., Feldman H., *Lancet Neurol.*, **2**, 539–547 (2003).
- Parihar M. S., Hemnani T., *J. Clin. Neurosci.*, **11**, 456–467 (2004).
- Rao A. A., Sridhar G. R., Das U. N., *Med. Hypotheses*, **69**, 1272–1276 (2007).
- Vassar R., *Adv. Drug Deliv. Rev.*, **54**, 1589–1602 (2002).
- Yan R., Bienkowski M. J., Shuck M. E., Miao H., Tory M. C., Pauley A. M., Brashier J. R., Stratman N. C., Mathews W. R., Buhl A. E., Carter D. B., Tomasselli A. G., Parodi L. A., Heinrikson R. L., Gurney M. E., *Nature (London)*, **402**, 533–537 (1999).
- Nes W. D., Norton R. A., Crumley F. G., Madigan S. J., Katz E. R., *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 7565–7569 (1990).
- Goad L. J., Akihisa T., "Analysis of Sterol," 1st ed., Chapman & Hall, U. K., 1997, p. 403.
- Ida Y., Satoh Y., Ohtsuka M., Nagasao M., Shoji J., *Phytochemistry*, **35**, 209–215 (1993).
- Tan J., Bednarek P., Liu J., Schneider B., Svatos A., Hahlbrock K., *Phytochemistry*, **65**, 691–699 (2004).
- van Tilburg E. W., van der Klein P. A., von Frijtag Drabbe Künzel J., de Groote M., Stannek C., Lorenzen A., IJzerman A. P., *J. Med. Chem.*, **44**, 2966–2975 (2001).
- Hara S., Okabe H., Mihashi K., *Chem. Pharm. Bull.*, **34**, 1843–1845 (1986).
- Ellman G. L., Courtney K. D., Andres V. Jr., Featherstone R. M., *Biochem. Pharmacol.*, **7**, 88–95 (1961).

- 42) Cornish-Bowden A., *Biochem. J.*, **137**, 143—144 (1974).
- 43) Mukherjee P. K., Kumar V., Houghton P. J., *Phytother. Res.*, **21**, 1142—1145 (2007).
- 44) Jung M., Park M., *Molecules*, **12**, 2130—2139 (2007).
- 45) Conforti F., Rigano D., Menichini F., Loizzo M. R., Senatore F., *Fittoterapia*, **80**, 62—67 (2009).
- 46) Okazaki R., Kiyota H., Oritani T., *Biosci. Biotechnol. Biochem.*, **64**, 1444—1447 (2000).
- 47) Min B. S., Nakamura N., Miyashiro H., Kim Y. H., Hattori M., *Chem. Pharm. Bull.*, **48**, 194—200 (2000).
- 48) Keane S., Ryan M. F., *Insect Biochem. Mol. Biol.*, **29**, 1097—1104 (1999).
- 49) Perry N. S., Bollen C., Perry E. K., Ballard C., *Pharmacol. Biochem. Behav.*, **75**, 651—659 (2003).
- 50) Miyazawa M., Watanabe H., Kameoka H., *J. Agric. Food Chem.*, **45**, 677—679 (1997).
- 51) Sauvaître T., Barlier M., Herlem D., Gresh N., Chiaroni A., Guenard D., Guillou C., *J. Med. Chem.*, **50**, 5311—5323 (2007).
- 52) Choudhary M. I., Shahnaz S., Parveen S., Khalid A., Mesaik M. A., Ayatollahi S. A., Atta-ur-Rahman, *Chem. Biodivers.*, **3**, 1039—1052 (2006).
- 53) Harvey A. L., *Pharmacol. Ther.*, **68**, 113—128 (1995).